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Salicylic Acid Pathway Changes in Barley Plants Challenged with either a Biotrophic or a Necrotrophic Pathogen

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The biotrophic *Blumeria graminis* (*Bg*) and the necrotrophic *Cochliobolus sativus*; (*Cs*) are economically important fungal pathogens of barley globally. To better understand barley mechanisms to resist these pathogens, changes in salicylic acid (SA) and its responsive genes particularly the pathogenesis related *PR1, PR2, PR3* and *PR5* were evaluated using qRT-PCR across four time points post infection. Data showed that SA contents significantly increased ($P = 0.001$) in infected plants of both resistant and susceptible genotypes 24 h post inoculation in comparison with non-infected controls. In addition, time-course tests revealed a notable contradiction in the defense-related genes expression patterns between barley and *Bg* and *Cs* interactions, showing that expression patterns of the same defense-associated genes were altered in adaptation to different pathogens. *PR1* and *PR2* genes were highlyactivated inresistant plants infected with the necrotrophic pathogen *Cs* rather than of the biotrophic one. The uniformity in barley defense response mechanisms could be in convention with the well-accepted notion that these responses are high intense in the resistant genotype. Our work provides useful information on the expected role of SA pathways in barley towards biotrophic and necroptrophic pathogens with different lifestyles.

Keywords: barley, powdery mildew, spot blotch, defense response, PCR (qPCR), salicylic acid

Introduction

Powdery mildew caused by the obligate biotrophic pathogen, *Blumeria graminis* f. sp. *hordei* (*Bg*), and spot blotch caused by the necrotrophic [*Cochliobolus sativus* (*Cs*) Drechs. ex Dastur] of barley are among the most devastating fungal diseases causing significant yield losses across the world (Kumar et al. 2002; Rsaliyev et al. 2017). Barley plants infected with *Bg* and *Cs* pathogens excited defense response which is regulated by various signalling pathways, including plant hormones such as salicylic acid and pathogenesis-related (*PR*) proteins (Kogel and Langen 2005; Jawhar et al. 2017a,b). However, our understanding of the pathosystems mediating barley infection by these two biotrophic and necroptrophic fungi is still limited.

Salicylic acid (SA) is a key defense signal molecule against plant pathogens, and its production can be changed with the expression profiling of responsive marker genes

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(Alvarez 2000; Haffner et al. 2014). Additionally, discovery of SA targets and the understanding of its molecular modes of action in physiological processes could help in the dissection of the complex SA signalling network, confirming its crucial role in both plant health and disease (Zhang et al. 2016).

A notable number of defense-related genes are activated during plant pathogen interactions (Nayanakantha et al. 2016), and SA levels often increase and induce the expression of several pathogenesis-related proteins and initiate the development of systemic acquired resistance and hypersensitive response (Alvarez 2000). However, the molecular events involved in SA signaling are not yet fully understood. Quantitative PCR (qPCR) is considered to be an effective method of detection since it allows the measurement of the relative expression level of a particular transcript and determines its expression after exposure to a specific alteration, such as infection by a pathogen (Derveaux et al. 2010).

The present work aimed at evaluating the changes in SA andits responsive pathogenesis related genes, *viz. PR1*, *PR2, PR3* and *PR5* during barley interaction with either abiotrophic (*Bg*) or a necrotrophic (*Cs*) pathogen deploying a qPCR approach.

Materials and Methods

Plant material and experimental design

The most resistant Banteng and the universal susceptible control cv. WI2291 of barley (*Hordeum vulgare* L.) to all *Bg* and *Cs* isolates available for more than 10 years (Arabi and Jawhar 2004; 2012) were used in this study. Seeds of each genotype were planted in plastic boxes (60 × 40 × 8 cm) filled with sterilized peatmoss with three replicates for each pathogen, and each experimental unit consisted of 10 seedlings. They were placed in a growth chamber in a randomized complete block design at temperatures 22 °C (day) and 18 °C (night) with a day length of 12 h and 90% relative humidity.

Infection with Cs

The virulent isolate (*Cs* 16) of *C. sativus* (Arabi and Jawhar 2004) was used in the experiments. Mycelia were grown on Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) for 10 days under 20 °C in the dark. Then, conidia were collected with 10 mL of sterile distilled water and the suspension was adjusted to 2×10^4 conidia/mL. A surfactant (polyoxyethylene-20-sorbitan monolaurate) was added (100 µL/L) to the conidial suspension to facilitate dispersion of the inoculum over the leaf surfaces. The primary leaves of 12-day-old seedlings were inoculated by uniformly spraying each plant with the conidial suspension using a hand-held spray bottle. Plants were covered for one night with plastic bags to increase humidity and plants were kept in the same greenhouse at 20 °C with a 16 h photoperiod. Infection responseswere scored according to the method described by Fetch and Steffenson (1999).

Inoculation with Bg

Inoculation was performed according to the protocol described by Chaure et al. (2000) on 12-day-old seedlings with conidiospores of a virulent *Bg* isolate by employing a soft hair brush to give about 10–20 conidia per one microscope field at ×150 magnification. Inoculated plants were placed under growth chamber conditions, while uninoculated control plants were transferred to a separate "clean" growth chamber and kept under plastic boxes to avoid infection with *Bg*. Infections were recorded according to the scale described by Moseman and Baenziger (1981).

SA quantification

SA was quantified in barley third leaf tissuesat 24, 48, 72 and 96 hours post inoculation (hpi). Three replicates were achieved for each time point. SA was measured using the method described by Trapp et al. (2014) with minor modifications. Extraction was achieved by adding 1.0 ml of ethylacetate, dichloromethane, isopropanol and MeOH: water (8:2) into each. The extract was centrifuged at 4 °C at 16,000 *g* for 5 min and the supernatants were removed into a new 1.5 micro-centrifuge tubes and dried in speed vac. After drying, 100 µl of MeOH was added to each sample, homogenized under vortex and centrifuged at 16,000 *g* and 4 °C for 10 min. SA was analyzed by a high performance liquid chromatography (HPLC) system (Agilent Technologies, Germany).

RNA isolation and cDNA synthesis

Third barley leaves from three individual biological replicates were collected at 24, 48. 72 and 92 hpi, and immediately frozen in liquid nitrogen, and samples from non-inoculated plants were collected as controls at the same time points. Non-inoculation was done by spraying plants with pathogen-free water. mRNA was extracted from samples with the Nucleotrap mRNA mini kit (Macherey-Nagel, Germany) following the manufacturer's recommendations. cDNA synthesis was carried out with the Quanti Tect Reverse Transcription Kit (Qiagen, Germany) following the manufacturer's instructions.

Quantitative real-time PCR (qPCR)

Gene expression was assayed in Step One Plus, 96 well using SYBR Green Master kit (Roche, USA). All cDNA samples, standards and controls (which were tested not to contain genomic DNA) were assayed in triplicate for each target gene in a single run. Four known defense-related *PR1*, *PR2, PR3* and *PR5* geneswere analyzed. The sequence information for all RT-PCR primers is given in Table 1. The threshold cycle (Ct) value was automatically determined for each reaction by the real time PCR system with default parameters. The standard curve calculation and data analysis was performed with Rotor-Gene Q software (Qiagen). Raw data (not baseline corrected) of fluorescence levels and the specificity of the amplicons were checked by qRT-PCR dissociation curve analysis

Table 1. Properties and nucleotide sequences of primers used in this study

Gene	Gene description	Accession No.	Sequence	Amplified fragment (bp)
EF1α	Elongation foctor-1 Alapha	AT1G07920	TGGATTTGAGGGTGACAACA	167
			CCGTTCCAATACCACCAATC	
PR1	Pathogen-related protein	AY005474	ACTACCTTTCACCCCACAACGC	182
			TTTCTGTCCAACAACATTCCCG	
PR2	Beta1,3-glucanase2	AT3G57260	TCATCCCTGAACCTTCCTTG	193
			GGGGCTACTGTTTCAAGCAA	
PR3	Basic chitinase	AT3G12500	GGGGCTACTGTTTCAAGCAA	187
			GCAACAAGGTCAGGGTTGTT	
PR5	Pathogen-related protein S	AT1G75040	GGAGACTGTGGCGGTCTAAG	197
			GCGTTGAGGTCAGAGACACA	

using StepOne™ Software v2.3. Seedlings inoculated with distilled water served as a control.

Data analysis

The fluorescence readings of three replicated samples were averaged, and the blank value (from no-DNA control) was subtracted. Relative expression levels were determined using the average cycle threshold (Ct). Average Ct values were calculated from the triplicate experiment conducted for each gene, with the ΔCT value determined by subtracting the average Ct value of genes from the Ct value of the *EF1α* gene. Finally, the equation $2^{-\Delta\Delta CT}$ was used to estimate relative expression levels, and the fold change in putative target gene expression levels was determined as described by Livak and Schmittgen (2001), with *EF1α* as a reference (housekeeping control) gene. Standard deviation was calculated from the replicated experimental data. The statistical analysis was conducted through the Tukey's test at the 0.05 level.

Results

Infection responses of the two selected barley genotypes against *Bg* and *Cs* are shown in Figure 1A, B. For *Bg*, our first notable results were the phenotypic differences between *Bg*–S and R genotypes. Infected leaves of susceptible WI 2291 plants showed the typical small, yellow spots of *Bg* at 4 dpi compared with the control (non-inoculated) genotypes. Several days later a white fluffy fungus can be seen in these spots (data not shown). The resistant genotype Banteng, in contrast, showed normal growth in comparison to WI 2291 as well as the uninoculated plants even at 14 dpi. The results are in agreement with our observations under natural field conditions during the past several years (Arabi and Jawhar 2012). On the other hand, the level of barley resistance to *Cs*, as assessed by

lesion density and area under the disease progress curve under controlled conditions. However, the disease symptoms (presence of necrosis and chlorosis) were always more severe in the highly susceptible cultivar WI2291 (data not shown), these are in line with our previous work (Arabi and Jawhar 2004). In order to evaluate the biochemical and physiological changes in barley responses to *Bg* and *Cs* infection, the total SA content and the induction of four important defense related genes *viz.*, *PR1, PR2, PR3* and *PR5* were assayed.

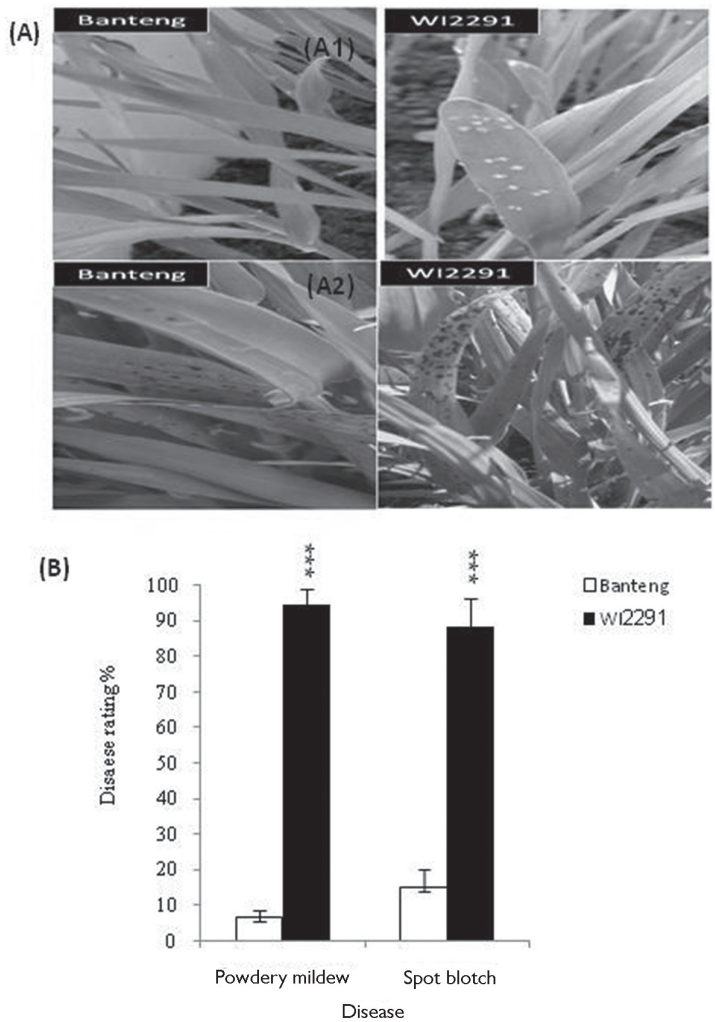

Figure 1. A: Disease symptoms on the barley resistant cv. Banteng and susceptible cv. WI2291 by (A1) powdery mildew according to the scale of Moseman and Baenziger (1981), and by (A2) spot blotch according to the scale of Fetch and Steffenson (1999). B: Frequency of disease reactions incited on the barley resistant cv. Banteng and susceptible cv. WI2291, 14 days after powdery mildew and spot blotch infection. Significance at ***$P<0.001$ between the two genotypes for each disease

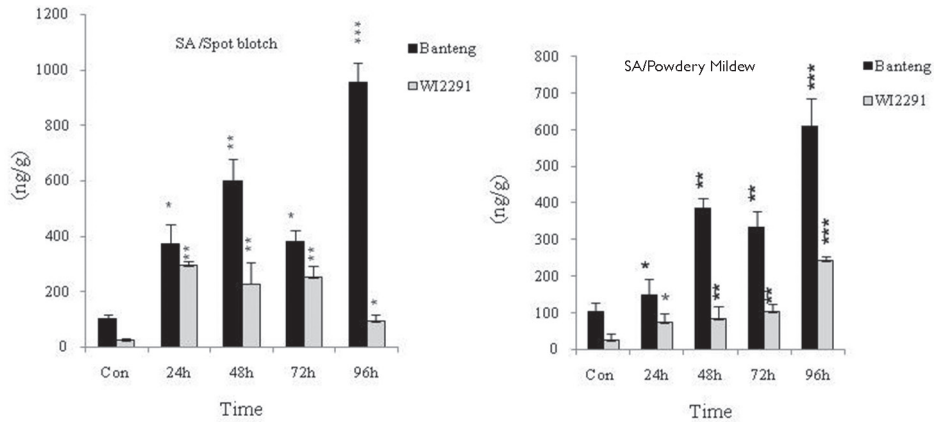

Figure 2. Quantification of total salicylic acid in barley leaves (cv. Banteng and cv. WI2291) 6 days post inoculation with *Bg* and *Cs*. Error bars are representative of the standard error (mean ± SD, $n = 3$). Significance at *P<0.05; **P<0.01 and ***P<0.001 within each genotype during different periods comparing with the control

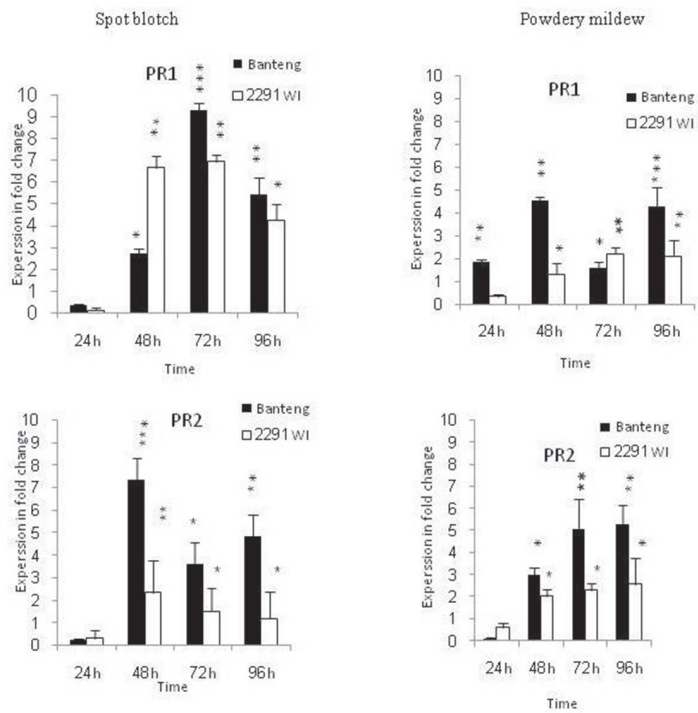

Figure 3.

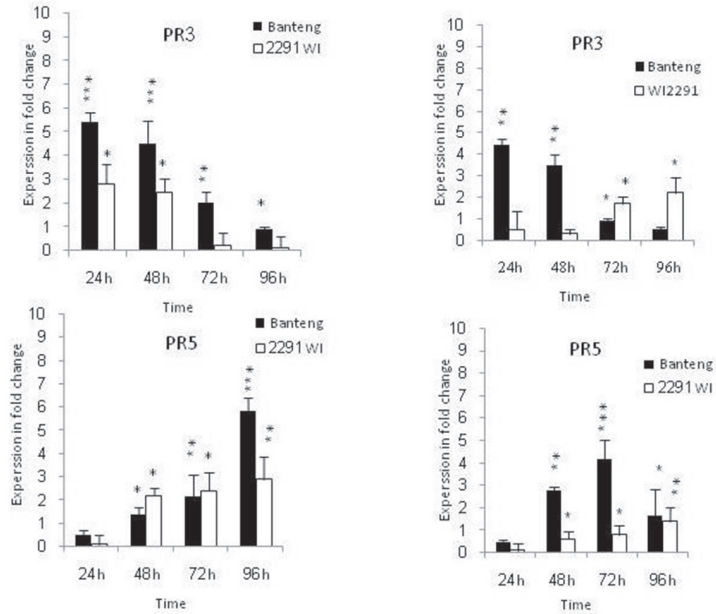

Figure 3. Relative expression profiles of marker genes in the resistant genotype Banteng and in the susceptible genotype WI2291during the time course following *Bg and Cs* infections. Error bars are representative of the standard error (Mean ± SD, $n = 3$). Data are normalized to Elongation factor 1α (EF-1α) gene expression level (to the calibrator, Control 0 h, taken as 0). Significance at *P < 0.05, **P < 0.01 and ***P < 0.001 within each genotype during different periods comparing with the control

The results showed significant changes ($P = 0.001$) in SA and its responsive pathogenesis related gene safter infection with both pathogens at 24, 48, 72 and 96 hpi in barley resistant and susceptible genotypes, and values were consistently higher in the resistant one (Figs 2 and 3). Data showed that SA level contents increased 24 hpi in leaves of the both genotypes as compared with non-inoculated plants, and the resistant genotype had higher levels than the susceptible one at each time point investigated (Fig. 2). This SA could be consideredto have an important role in the SA dependent signaling pathways during *Bg* and *Cs* infections. In addition, the remarkable expression of SA-related genes in the resistant 'Banteng' and susceptible 'WI2291' genotypes can be speculated that the positive role of SA in inducing systemic defense in barley upon *Bg* and *Cs* infection is less direct than other defense regulatory phytohormones.Moreover, *PR1* and *PR2* genes were highly expressed with 9.32 and 3.61-fold increases 72 hpi in resistant plants infected with necrotrophic pathogen *Cs* rather than of the biotrophic one (Fig. 3).

Discussion

Our results are supported by previous works indicating that the selected marker genes, which are relevant to the SA pathway, showed similar expression patterns in genotypes

with varying susceptibility, suggesting that the SA signaling pathway is efficiently activated in the susceptible cultivar, which is in line with Zhang et al. (2016). However, increasing SA levels post *Bg* and *Cs* infection in a plant does not mean that SA is the main cause of barley resistance since the infection by both pathogens may induce plant responses regulated by other hormones such as jasmonic acid, ABA and ethylene (Häffner et al. 2014). SA accumulation has been documented as a credible marker in defense responses that are associated with redox homeostasis and hypersensitive cell death (Dong 2004), which might be due to the fact that SA binds and inhibits catalase activity (Shim et al. 2003).

Data demonstrated that selected genes in both barley genotypes displayed a different expression at $P = 0.001$, that were associated with the activation of SA levels. This might be due to the fact that *C. sativus* has an initial biotrophic stage during disease development, which would reflect a pathogenic strategy to cope with plant cell death-associated defense (Gupta et al. 2018). Interestingly, some genes were associated with a multi-gene resistance which removes the current credence that similar mechanisms are involved in defense responses to biotrophic and necrotrophic pathogens.

On the other hand, the *PRs* roles for encoding 1,3-β-glucanase that hydrolyses the β-*O*-glycosidic bond of β-glucan in plant cell walls which causes cell wall loosening and expansion have been well documented (Akiyama et al. 2009). This effect could be the cause of barley cell wall leakage during *Bg* and *Cs* infections. However, PR1 being a β-1, 3-glucanase, this protein is involved in the degradation of fungal cell walls, being required for the hydrolysis of glucan (Van loor et al. 2006). In this work, PR1 transcripts peaked at 48 and 72 hpi in the resistant cv. Banteng for the *Bg and Cs* respectively, and presumably is targeting secondary hyphal growth for these both pathogens.

Furthermore, previous works indicate that PR genes are associated with MLO powdery mildew resistance (Tayeh et al. 2015) and so should be further investigated to support the current results. It is of a particular interest to highlight the induction of defense-related genes at early stages of infection. It has been well established that biotrophic *Uromycesvignae* and hemibiotroph *Mycosphaerella graminicola* have suppressed the host defenses for parasitize that invaded host cells during the biotrophic phase (Panstruga 2003; Doehlemann et al. 2008).

In conclusion, this work demonstrated that significant increases in SA and its responsive pathogenesis related genes *PR1, PR2, PR3* and *PR5* were found at early stages upon barley challenged with biotrophs and necrotrophs, with values being higher in the resistant genotype. The results could suggest that SA signaling pathways probably work together in the activation of defense responses against both pathogens. The uniformity in the defense response mechanisms could be in convention with the well-accepted notion that these responses are high intense in resistant genotypes. In addition, the data indicated that not only SA is paramount for the defense responses induction, but in the obscurity of pathogen attack, SA might sustain basal levels of genes connected with barley resistance responses and keep the defense system primed against biotrophic and necrotrophic pathogens.

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